parallel to the surface[8] and it is expected to produce a dioxetane stabilized by the electron from $O_2^-{}_{(ads)}$. The evolution of CO and H_2 can be explained in terms of the scission of the dioxygen bridge and the C-C bond of dioxetane; the decomposition of the fragments above 423 K.

The decrease in the depth of the insulating layer at 473 K and 573 K can be explained as follows; the adsorbed atomic oxygen $O^-{}_{(ads)}$ reacts with C_2H_4 at those temperatures and the electron trappped at O^- moves to ZnO. These results are consistent with EPR measurements of the powder ZnO.[4] Mass spectrometric analysis showed the formation of CO_2 and H_2O at 473 K and 573 K.

Acknowledgments. This work was supported by Korea Science and Enginnering Foundation (931-0300-004-2).

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8. EPR spectrum of $^{17}O^{16}O^-$ shows one set of hyperfine splitting with $a_{yy}=80$ G.

High Performance Liquid Chromatographic Determination of Residual Sulfonamide Antibacterials in Meat using Alumina Cartridge

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Received January 4, 1994

Sulfonamide antibacterials (SAs), a kind of the synthetic antibacterials that have been used since the early part of the 1900's, are widely used for the treatment and prevention of disease of livestock and cultured fish, and the increase of feed efficiency[1]. Residues of these drugs in food derived from treated animals could pose a health threat to consumers, and the constant exposure of some microorganisms to these drugs may manifest itself in the development of drug-resistant strains. Also recent evidence has implicated some SAs as a possible carcinogen.[2]

When considered to increase the consumption of the livestock products day by day, a simple, rapid and reliable method for multiresidue analysis of SAs is urgently required. But national official method of USA[3], Japan[4], etc., including Korea[5] are not suitable for routine analysis because of the long analysis time, especially complicated clean-up procedure and being the individual determination method with respect to each SA.

Today, the various determination methods of SAs[2,6,7] has been developed and utilized as the development of the analytical instruments, but a simple and rapid clean-up procedure is a key point of routine analysis.

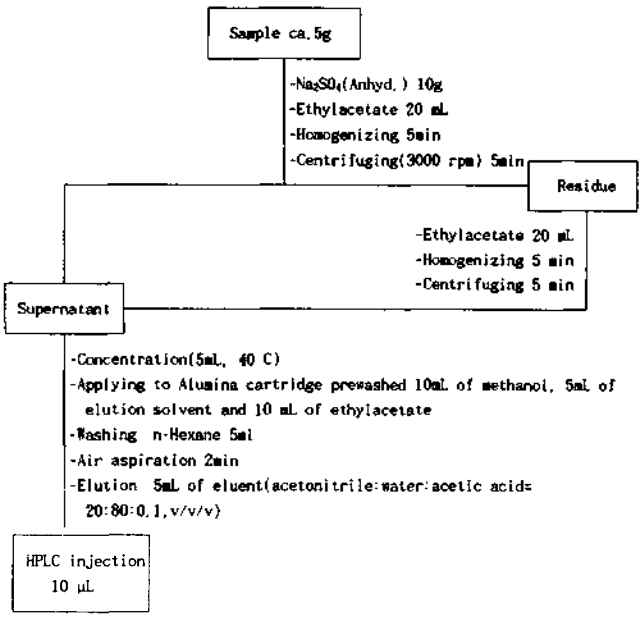
In order to simplify the clean-up procedure, prepacked cartridges have been utilized[8-11], among which the reversed phase type are the most frequently used. However, the normal phase and ion exchange types are more suitable than the reverse phase type because reverse phase cartridges need some time-consuming pretreatments such as evaporation of the organic solvents and elimination of fat from the extracts for clean-up of extracted SAs, whereas normal phase type cartridges do not require such pretreatments.[11]

Therefore, in this paper, we tried to establish a clean-up procedure using the normal phase type Alumina cartridge that not reported until now, and applied to meat for multiresidue ananlysis as the main target of five SAs established the tolerence limits in the Korean Code of Food Standards.[5]

Experimental

Apparatus. A HPLC system (Waters, Millipore Corp., Millford, MA, USA) eqipped with a constant flow pump (510 Solvent Delivery System) was used with a UV detector (486 Tunable Absorbance Detector) operated at 270 nm. The operating condition was described in Table 1. Alumina (Part No. 20500) and Florisil (Part No. 20525) cartridges were purchased from Waters (Millipore Corp., Millford, MA, USA).

Chemicals. Sulfadiazine (SDZ), sulfamerazine (SMRZ), sulfamethazine (SMTZ), sulfamonomethoxine (SMMX), sulfadimethoxine (SDMX) and sulfaquinoxaline (SQX) were ob-



Scheme 1. Flow chart of extraction and clean-up procedure for SAs.

Table 1. Operating Condition of HPLC for SAs

Column	μBondapak C18 (3.9×300 mm, 10 μm)
Mobile Phase	Acetonitrile : Water : Acetic acid (20 : 80 : 0.1)
Column Temperature	Room Temperature
Flow rate	1.5 mL/min
Detector	UV 270 nm (0.01 AUFS)
Injection volume	10 μL

tained from Sigma (St. Louis, MO, USA) and acetonitrile, methanol and n-hexane were used HPLC grade of Fisher Scientific (Fair Lawn, NJ, USA). Anhydrous sodium sulfate, ethylacetate, acetic acid and oxalic acid were of analytical grade and water of HPLC analysis were triple-distilled water polished by E-PURE (Barnsted, Iowa, USA) water purification system.

Samples. Beef, pork, chicken and internal organs of cattle, swine and fowls were obtained from meat shops and local markets in Chuncheon, Sokcho and Taebaek, Kangweon province, and kept frozon in refrigerator after chopping.

Standard solution and Calibration. Each standard (20 mg) was weighed accurately into 20 mL volumetric flask, and added 5 mL of water and 5 mL of acetonitrile, and diluted to volume with acetonitrile. Calibration curve was drawn from the obtained peak areas after injected 5 ng, 10 ng, 25 ng, and 50 ng of the properly diluted standard solution in HPLC.

Clean-up procedure. Chopped sample (5 g) was exactly weighed in 50 mL centrifuge tube and added 10 g of anhydrous sodium sulfate, blended with 20 mL of ethylacetate for 5 min using a homogenizer (AM-7, Nihonseiki, Japan) and centrifuged for 5 min (Centrifuge, Yamamoto, Japan, Max : 4000 rpm). And the supernatant was decanted in an evarporating flask. The extraction with ethylacetate was repeated once more, combined with two extracts and concentrated to about 5 mL at 40℃. Concentrated extract was applied to an alumina cartridge prewashed with 10 mL of methanol, 5 mL of eluent (acetonitrile : water : acetic acid = 20 : 80 : 0.1, v/v/v) and 10 mL of ethylacetate. Then the cartridge was washed with 5 mL of n-hexane and dried by air-aspiration for 2 min. SAs were eluted from cartridge with eluent, and analyzed by HPLC according to the operating condition described in Table 1. In whole procedure, the flow rate was adjusted 2-3 mL/min by aspirator or vaccum pump.

Recoveries and Sample analysis. Standard solutions were added to beef, pork, chicken and Israeli carp, in which SAs have not been contained, to be 1.0-2.0 μg/mL levels, and after the samples were allowed to stand for several minutes, treated by the clean-up procedure. Thereafter 10 μL of the elute were injected into the HPLC and then the recoveries was calculated from the peak areas of the chromatograms. Also, 5 samples of beef, 5 samples of pork, 5 samples of chicken and every 2 samples of beef, pork and chicken's internal organs were analyzed by the same clean-up procedure and HPLC condition.

Results and Discusstion

HPLC condition. Generally, TLC is unsuitable for precise determinations and GC requires complicated treatment to derivitize SAs[5,11], but HPLC enables SAs to be determined rapidly and high sensitively without any additional treatment such as GC, so we tried to select optimal conditions of HPLC for simultaneous analysis for various SAs. The various HPLC chromatographic methods have been used for SAs analysis[2,10-12], but in this paper, run type was selected isocratic mode and the mobile phase was used a combination of acetonitrile, water and acetic acid (20 : 80 : 0.1, v/v/v). The flow rate was 1.5 mL/min and the monitoring wavelength was adjusted 270 nm to be considered that the UV maximum wavelength range was 265-275 nm and to simultaneous determination of several SAs and other synthetic antibacterials for the future.[7,12]

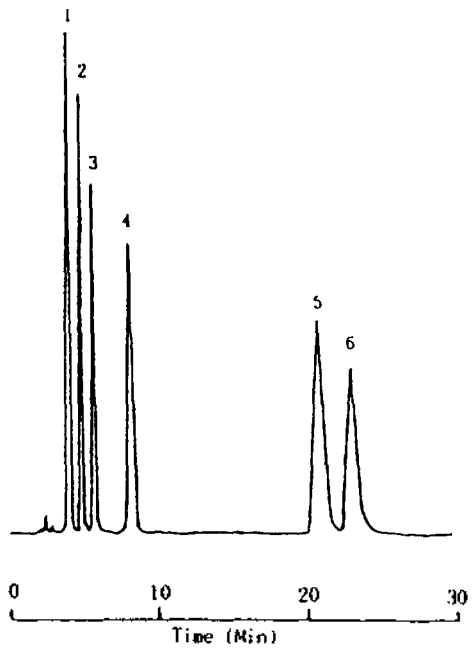


Figure 1. Typical HPLC chromatogram of SAs: 1. SDZ, 2. SMRZ 3. SMTZ, 4. SMMX, 5. SDMX, 6. SQX.

Figure 1. shows the typical chromatogram for SAs separated by the operating condition described in Table 1. The retention times of each SA are the followings; SDZ 4.1 min, SMRZ 5.0 min, SMTZ 5.8 min, SMMX 8.3 min, SDMX 21.1 min and SQX 23.4 min, respectively. Those results showed good resolution and resemblance of the early reported results.[2,7,11]

Calibration curve. The peak areas obtained from chromatograms of HPLC were evaluated as a function of the injection quantities (ng) by the least squares best fitting method. For SDZ, SMRZ, SMTZ, SMMX, SDMX and SQX, the slopes were 5204, 5278, 4726, 5284, 4963 and 3694 and the intercepts were −1916, −1179, −1510, −729, −3306 and −4191 and the correlation coefficients were 0.9999, 0.9999, 0.9999, 0.9999, 0.9998 and 0.9995, respectively. The good linearity and correlation were investigated in the calibration range.

Clean-up procedure. Methanol, acetone, acetonitrile, methylene chloride, chloroform, ethylacetate and various combinations of them have been widely used for the extraction of SAs from various biological samples[1,4,13]. But only me-

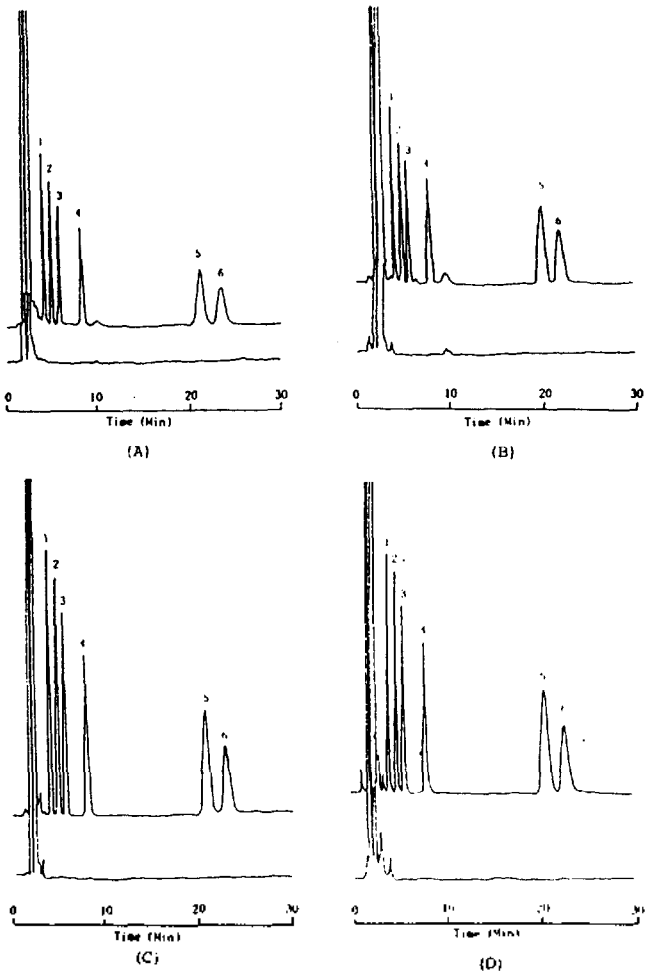


Figure 2. HPLC Chromatograms of spiked and unspiked (A) beef, (B) pork, (C) chicken and (D) Israeli carp: 1. SDZ, 2. SMRZ 3. SMTZ, 4. SMMX, 5. SDMX, 6. SQX.

thylene chloride, chloroform and ethylacetate among the these solvents can be considered as the extracting solvents for normal phase type of cartridge. However, methylene chloride and chlorofrom were not suitable for decantation of the supernatant after centrifugation because of their gravity, *i.e.* suspended particles or emulsion.[11]

On the basis of the above considerations, ethylacetate was used as the extracting solvent of SAs from samples. The volume of extracting solvent was adjusted to 40 mL as reported in the literature.[11] The extracts were concentrated to 5-10 mL and then directly applied to Alumina cartridge. The concentration step was necessary to obtain a better recovery.

The cartridge to be used in this method is required to possess both the ability to retain SAs with an organic solvent and then to release SAs with a suitable eluent. Two commercially available normal phase type prepacked cartridges (Alumina and Florisil) were compared. Alumina cartridge showed a better ability to retain various SAs with ethylacetate than Florisil cartridge, and much less impurity peaks were observed with Alumina cartridge.

In order to determine analytes in the eluent from the cartridge by HPLC, the eluent is usually evaporated and the residue is dissolved in a suitable solution before injection into the HPLC system. However, these treatments are not desirable for simple and rapid determination, so we wished to elute the SAs with a small volume of the mobile phase, because the eluent can be injected into HPLC system without any treatment. The elution patterns of SAs from cartridge were investigated using the mobile phase as the eluent. The SAs were eluted from the cartridge with the eluent and the SAs in each collected fraction (5 mL) were determined. As SAs were sufficiently recovered in the first fraction (98.8% in the first fraction, 1.1% in the 2nd fraction, 0.4% in the 3rd fraction), we used 5 mL of the mobile phase for elution of SAs from the cartridge. The flow rate was adjusted to 2-3 mL/min in whole procedure and after washing n-hexane, the cartridge was fully air-aspirated to prevent from emulsifing the eluent.

Table 2. Recoveries of SAs in meat according to the present method at the level 1.0-2.0 μg/mL (unit : %)

	SDZ	SMRZ	SMTZ	SMMX	SDMX	SQX	Average ± S.D
Beef	87.0	81.4	80.8	90.4	87.9	72.4	83.3± 6.5
Pork	75.0	76.0	76.8	80.4	85.0	76.2	78.2± 3.8
Chicken	86.5	76.7	87.2	81.8	77.7	78.9	81.5± 4.5
Israeli Carp	91.3	90.3	92.5	88.0	87.6	77.1	87.8± 5.6

Typical chromatograms of spiked and unspiked beef (A), pork (B), chicken (C) and Israeli carp (D) treated by the present clean-up procedure were shown in Figure 2. In Figure 2, all chromatograms ((A), (B), (C), (D)) show good resolutions and well separated peaks enough to be used for quantitative analysis of SAs.

Recoveries and Sample analysis. The recoveries of SAs were investigated at the addtion level of 1.0 μg/mL for beef and pork and 2.0 μg/mL for chicken and Israeli carp using our clean-up procedure. As shown in Table 2, satisfactory recoveries (72.4-92.5%, ave. 82.7%) were obtained. As compared with the recoveries of Murayama, *et al.*[14] (64.7-95.4 %, ave.: 75.5%) and Jung, *et al.*[7] (73.5-88.2%, ave.: 81.6%), these results are excellent.

Also seven samples among the 21 commercially available samples are containing more than one kind of SAs. No SAs were detected in pork and chicken and two SAs such as SMTZ (0.66 μg/mL) and SDMX (0.64 μg/mL) were detected in one beef sample. But many SAs were detected in mostly internal organ samples: SMRZ (0.03-0.09 μg/mL), SMTZ (0.20-2.40 μg/mL) and SDMX (1.03 μg/mL) in the internal organ of beef, SMRZ (0.27 μg/mL) and SMTZ (0.32-0.85 μg/mL) of pork, and SMTZ (0.46 μg/mL) and SMMX (0.57 μg/mL) in a internal organ of chicken. These results coincide with the reports of high residuality in internal organs[15].

In conclusions, a method of the determination of residual SAs in meat and fish was established using a combination of HPLC and clean-up procedure with alumina cartridge. The method is not only simple and rapid, but also permit the multiresidue analysis of SAs with good accuracy and reproducibility compared with offical method in the Korean Code of Food Standards[5] or the solvent extraction methods.[1,7,12,14]

And as there is the possibility of the simultaneous determination with SAs for several other synthetic antibacterials containing ethopabate, this method, extended and complemented a little more, is considered to be used as the official method.

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Synthesis of Fosfazinomycin Derivatives

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Received January 21, 1994

In the previous papers, we reported synthesis of Fosfazinomycin A and B.[1,2] Aminophosphonic acids and their derivatives have attracted attention because of their antibiotic, herbicidal, pesticidal, anticancer and enzyme inhibitory activities, and particulary their structural similarity to the biologically important amino acids. Since 2-aminophosphonic acid (2-AEPn) was isolated from sheep rumen in 1959 by Horiguchi and his coworkers,[3] many aminophosphonic acids and their derivatives have been discovered in living organism. Aminophosphonic acids are also discovered in mammalian tissues like human muscale, sheep liver, and ox brain.[4-10] Its concentration in human tissues was higher in heart and skeletal muscale than in liver and brain. Recently, incorporated with synthetic derivatives, biological activity of aminophosphonopeptides were widely investigated. Among various disciplines neurochemistry and neuropharmacology are the most brisk areas concerning the activity of aminophosphonic acid.[11] Fosfazinomycin, formerly called AM 630, is a new antifungal substance isolated from the fermentation broth of *Steptomyces lavendofoliae*.[12-15]

In this paper, the authors wish to report the synthesis of Fosfazinomycin derivatives **1(a)-1(d)**, dialkyl arginylvalyl-*N*-methylhydrazinophosphate, as shown in Scheme 1. Dialkyl chlorophosphate (**2**) was reacted with methylhydrazine to give dialkyl *N*-methylhydrazinophosphate (**3**). The peptide **6** was prepared by coupling of *N*-carbobenzyloxy-*N*-nitroarginine (**4**) with methyl valinate (**5**). *N*-Carbobenzyloxy-*N*-nitroarginylvaline (**7**) was reacted with dialkyl *N*-methylhydrazinophosphate (**3**) to give a coupled product of dialkyl *N*-carbobenzyloxy-*N*-nitroarginylvalyl-*N*-methylhydrazinophosphate **8(a)-8(d)**. The deprotection of **8** by hydrogenation yielded Fosfazinomycin derivatives **1(a)-1(d)**. In conclusion, Fosfazinomycin derivatives **1(a)-1(d)**, a new kind of phosphorus compunds, were synthesized efficiently in 5 steps with 23-29% overall yields.

Experimental Section

All reactions were carried out with the precaution for rigorous exclusion of air and moisture. The solvents, ether and THF, were purified by refluxing for several hours in the presence of sodium metal and benzophnone followed by distillation under nitrogen prior to use. Melting points were measured by Mettler F61 melting point apparatus. IR spectra were recorded with Beckmann acculab TMI spectrometer, and proton NMR spectra were taken on Varian EM-360 (80 MHz) spectrometer with TMS as an internal standard. Low pressure hydrogenation was carried out with Parr instrument hydrogenator.

Dimethyl *N*-methylhydrazinophosphate (3a). To a solution of dimethyl chlorophosphate (**2a**) (4.64 g, 0.032 mol) in dry THF (20 m*l*), a solution of methylhydrazine (1.68 m*l*, 0.032 mol) and pyridine (2.6 m*l*) was added slowly under nitrogen at −78℃. The mixture was stirred for 4 hr at −78 ℃ and 8 hr at room temperature. A few m*l* of H_2O was added to the solution, and the resulting mixture was extracted with ether and ethyl acetate successively. The combined organic layers were dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was chromatographed on a silica gel column using ethyl acetate and hexane (1 : 1, v/v) as an eluent to give an oily product in 66% yield.

Anal. Calc. for $C_3H_{11}N_2O_3P$; C, 23.38; H, 7.14; N, 18.19;